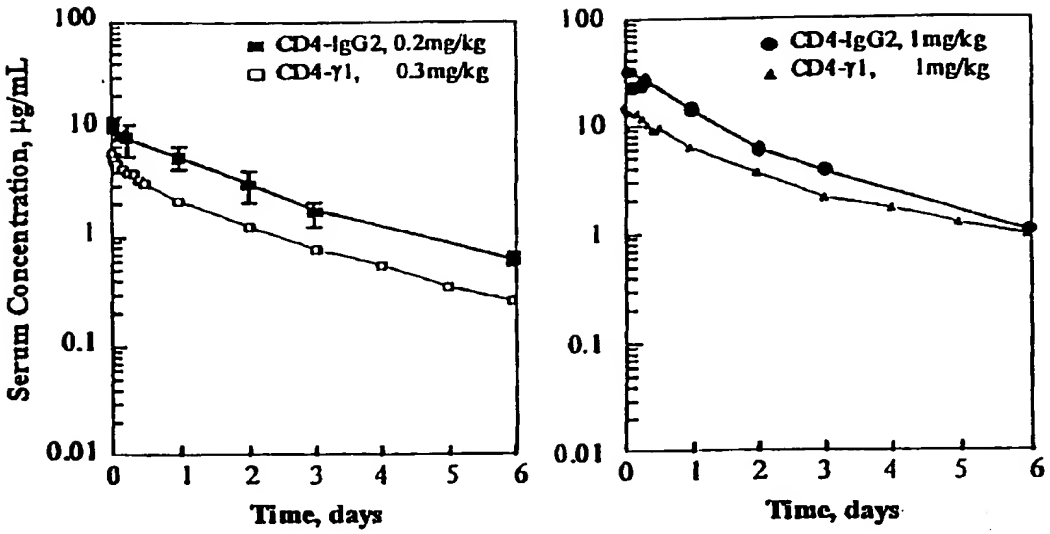
CD4-IgG2, 0.2mg/kg
CD4-γ1, 0.3mg/kg
Serum Concentration, µg/mL
100
10
1
0.1
0.01
0 1 2 3 4 5 6
Time, days
CD4-IgG2, 1mg/kg
CD4-γ1, 1mg/kg
100
10
1
0.1
0.01
0 1 2 3 4 5 6
Time, days

each well. Cultures are maintained at 37°C for 48 hours, and syncytia are counted using light microscopy at 200x. Table IV lists the concentration of CD4-γ2 and sCD4 required to give 50% inhibition of syncytia formation (IC_{50}):

Molecule	IC_{50}	
	(μg/ml)	nM
sCD4	9.19	200
CD4-γ2	4.03	40

Table IV: Syncytium inhibition data

These results demonstrate that CD4-γ2 inhibits HIV-1 envelope-mediated cell fusion and is more effective than sCD4 on the basis of mass or molarity.

iii) HIV-1 Neutralization Studies:

CD4-γ2 has been tested for ability to neutralize HIV-1 *in vitro*, using a laboratory-adapted strain and a primary isolate of HIV-1. These assays were performed as previously described[28]. Briefly, 50 tissue culture infectious doses of the laboratory-adapted strain HIV-1_{LAI} or the primary isolate HIV-$1_{JR\text{-}CSF}$ were incubated with serial 5 fold-dilutions of CD4-γ2 or sCD4 for 30 min. at 37°C. The mixtures were then added to 2 x 10^6 PHA-activated normal donor peripheral blood mononuclear cells. The cultures were washed on day 1, and the cellular supernatants were assayed for p24 core antigen expression on day 7. The percent neutralization was determined by comparing p24 antigen concentration in treated and untreated cultures. The concentrations of CD4-γ2 and sCD4 giving 90% neutralization (IC_{90}) are shown in Table V.

Molecule	IC_{90} (μg/ml)	
	LAI	JR-CSF
sCD4	4.4	4.9
CD4-γ2	1.5	1.6

Table V: Concentrations of sCD4 or CD4-γ2 giving 90% neutralization of HIV-1_{LAI} and HIV-$1_{JR\text{-}CSF}$.

These results demonstrate that CD4-γ2 is biologically active and neutralizes these isolates of HIV-1 more potently than sCD4.

iv) Fc Receptor Binding Analysis:

CD4-γ2 incorporates the Fc portion of human IgG2 in order to minimize Fc-mediated function, including Fc receptor binding. Flow cytometry was used to analyze the binding of CD4-γ2 to the human monocytic cell line U937 that expresses FcRγI, FcRγII and FcRγIII. 1μg/ml of CD4-γ2 or control human IgG1 (Sigma) was incubated with U937 cells for 1 hour at 37°C, washed and incubated with FITC-goat anti-human IgG at 4°C for 30 minutes, washed and analyzed using a Becton-Dickinson FACScan. The results (not shown) demonstrated that whereas control human IgG1 avidly binds to Fc receptors on U937 cells, CD4-γ2 exhibits no measurable Fc receptor binding, eliminating a potential mechanism for CD4-γ2 binding to non-target cells.

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Effective Ex Vivo Neutralization of Human Immunodeficiency Virus Type 1 in Plasma by Recombinant Immunoglobulin Molecules

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We tested the ability of human monoclonal antibodies (immunoglobulin G1b12 [IgG1b12] and 19b) and CD4-based molecules (CD4-IgG2 and soluble CD4 [sCD4]) to neutralize human immunodeficiency virus type 1 directly from the plasma of seropositive donors in an ex vivo neutralization assay. IgG1b12 and CD4-IgG2, at concentrations from 1 to 25 μg/ml, were found to be effective at reducing the HIV-1 titer in most plasma samples. When viruses recovered from plasma samples were expanded to produce virus stocks, no correlation between the neutralization sensitivities to IgG1b12 and CD4-IgG2 of the in vitro passaged stocks and those of the ex vivo neutralizations performed directly on the plasma was observed. These differences could be due to changes in neutralization sensitivity that occur after one passage of the virus in vitro, or they could be related to the presence of complement or antibodies in the plasma. Furthermore, differences in expression of adhesion molecules on plasma-derived and phytohemagglutinin-activated peripheral blood mononuclear cell-derived viruses could be involved. These studies suggest that IgG1b12 and CD4-IgG2 have broad and potent neutralizing activity in both in vitro and ex vivo neutralization assays and should be considered for use as potential immunoprophylactic or therapeutic agents.

Recombinant soluble CD4 (sCD4), which represents the extracellular domain of the cell surface receptor for human immunodeficiency virus type 1 (HIV-1), was initially demonstrated to effectively block infection of laboratory strains of HIV-1 in vitro (11, 14, 17, 18, 23, 36, 40). However, it was later shown that primary isolates of HIV-1 are relatively resistant to neutralization by sCD4 (3, 10, 23) and that the sensitivity of primary HIV-1 isolates could be increased by repeated passage in vitro (10, 23). The clinical relevance of these findings became apparent when it was demonstrated that, at a concentration capable of neutralizing laboratory-adapted strains of HIV-1, sCD4 was ineffective at neutralizing HIV-1 directly from samples of patient plasma in an ex vivo format (10). This finding was correlated with relative ineffectiveness in therapeutic trials (9, 10). It has now been shown that primary HIV-1 isolates are relatively resistant not only to neutralization by sCD4 (3, 10, 23, 27, 32) but also to monoclonal antibodies (24, 25) and other CD4-based molecules (18, 24), compared with laboratory-adapted strains of HIV-1, a finding that has caused concern among those charged with developing immunotherapeutics and vaccines against HIV-1 (9).

Despite the relative resistance of primary HIV-1 isolates to neutralization, recent studies have shown that primary HIV-1 isolates can be neutralized in vitro by certain monoclonal antibodies, CD4-based molecules, and the sera of long-term nonprogressors from HIV-1 infection (1, 7, 8, 41). Most of the potent monoclonal antibodies, however, have not been tested for their ability to neutralize HIV-1 directly from plasma, as was done with sCD4. We therefore sought to study the ability of these newer molecules to neutralize HIV-1 directly from patient plasma samples in an ex vivo assay. The antibody products we chose to study were immunoglobulin G1b12 (IgG1b12), a human monoclonal antibody produced from a combinatorial phage display library (7, 33), and CD4-IgG2, a tetrameric human antibody prepared from a human IgG2 with replacement of each heavy- and light-chain variable region by the first and second domains of human CD4 (1). Both IgG1b12 and CD4-IgG2 recognize discontinuous epitopes overlapping the CD4-binding site (CD4-BS) on HIV-1 gp120 (1, 7, 33) and have been shown to effectively neutralize primary HIV-1 isolates in standard in vitro assays (1, 7). In our studies, controls included sCD4 and 19b, a human V3-specific antibody that recognizes determinants within the third variable region of HIV-1 gp120 (28, 35). Monoclonal antibody 19b was produced by B-cell transformation and cloning and has limited activity against primary isolates of HIV-1 (24, 41).

We first assessed the ability of IgG1b12, CD4-IgG2, and sCD4 to neutralize four distinct isolates of HIV-1 in a standard in vitro neutralization assay. The four HIV-1 isolates included a virus adapted to grow in transformed T-cell lines (LAI), a molecularly cloned isolate (JR-CSF) that demonstrates many of the characteristics of a primary HIV-1 isolate and will not grow in transformed T-cell lines (20), and two primary HIV-1 isolates (AD6 and WH91-330) that have been passaged twice in mitogen-stimulated peripheral blood mononuclear cells (PBMC) and will not grow in transformed T-cell lines (19, 42). IgG1b12, CD4-IgG2, and sCD4 were tested at concentrations from 0.006 to 100 μg/ml for their ability to neutralize a fixed inoculum of these viruses (200 50% tissue culture infective doses [$TCID_{50}$]) on phytohemagglutinin (PHA)-stimulated PBMC by previously described methods (8, 24, 41). Viral p24 antigen was measured by enzyme immunoassay (Abbott Laboratory, Abbott Park, Ill.) on days 4 to 7, and neutralization curves were generated (Fig. 1).

Consistent with previous observations (1, 24), sCD4 effectively neutralized LAI and JR-CSF (Fig. 1) with 50% inhibitory doses (ID_{50}) and ID_{90} of 0.024 and 0.39 μg/ml for LAI and 0.8

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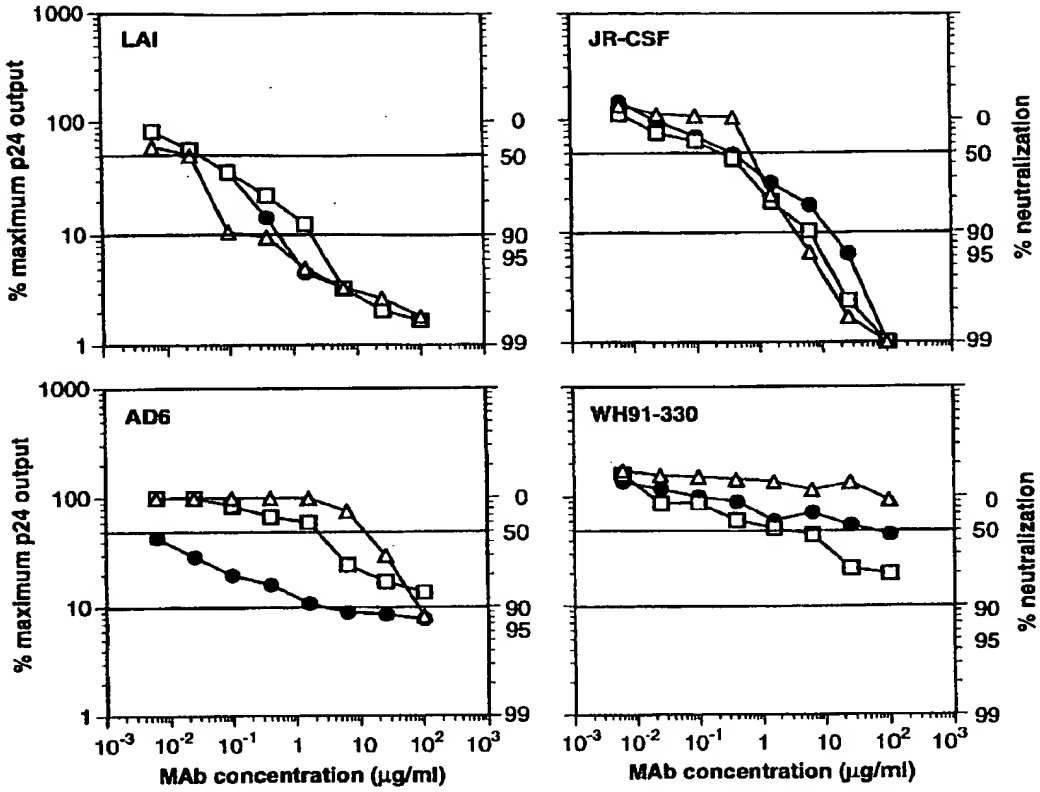

FIG. 1. In vitro neutralization of four HIV-1 isolates by CD4-IgG2, IgG1b12, and sCD4. CD4-IgG2 (□), IgG1b12 (●), and sCD4 (△) were tested for neutralization activity in vitro against a standard inoculum (200 $TCID_{50}$) of HIV-1 isolates, including LAI, JR-CSF, AD6, and WH91-330. Assays were performed on PHA-stimulated PBMC. The virus replication was monitored by measurement of p24 antigen on days 5 to 8. Assays were run in triplicate, and the mean values were plotted as the percentage of maximum p24 output against input concentration (in micrograms per milliliter). Horizontal lines represent 50 and 90% inhibition of p24 output. MAb, monoclonal antibody.

and 5.5 μg/ml for JR-CSF, respectively. However, sCD4 was not effective in neutralizing WH91-330 (<20% reduction in p24 production at 100 μg/ml) but was moderately effective in neutralizing the other primary isolate AD6, with an ID_{50} of 9 μg/ml and an ID_{90} of 90 μg/ml. Neither LAI nor JR-CSF showed significant differences in neutralization sensitivity to sCD4, IgG1b12, and CD4-IgG2. In contrast, primary isolates AD6 and WH91-330 were more sensitive to neutralization by IgG1b12 and CD4-IgG2 than by sCD4. While there was some loss in neutralization sensitivity to IgG1b12 and CD4-IgG2 when moving from laboratory strains to primary isolates, the greatest loss in sensitivity was to sCD4 (Fig. 1). These results confirm that primary isolates AD6 and WH91-330 are relatively resistant to neutralization, even by IgG1b12 and CD4-IgG2, but to a much lesser degree than they are resistant to neutralization by sCD4.

We next sought to test the ability of IgG1b12 and CD4-IgG2 to neutralize viruses that had not previously been subjected to passage, and possible selection, in vitro, by using a previously published method (10). All plasma samples used in this study were drawn from HIV-1-infected patients in the New York metropolitan area. Briefly, sCD4, 19b, IgG1b12, or CD4-IgG2 (final concentration, 25 μg/ml) was added to 24-well plates containing serial fivefold dilutions of HIV-1-infected plasma. The mixture was incubated with 2×10^6 PHA-stimulated PBMC from an uninfected donor. After 24 h, the cultures were washed extensively and cultured for 14 days. Viral replication was measured by the expression of p24 antigen in the culture supernatants by using a commercial enzyme immunoassay (Abbott) on days 7 and 14. An end-point titer of infectious HIV-1 in the presence or in the absence of added reagent was calculated (10, 16). A culture was considered positive if the p24 value was above 50 pg/ml.

Plasma samples from six donors were used to perform ex vivo neutralization assays. These HIV-1-infected plasma samples were selected on the basis of having an initial infectious titer of at least 250 $TCID_{50}$/ml and therefore were derived from patients in the later stages of HIV-1 infection. The infectious titer of HIV-1 in the plasma samples in the presence or in the absence of each monoclonal product is shown in Fig. 2. With this assay we were unable to reproducibly measure a fivefold or smaller reduction in infectious titer within any given plasma sample (data not shown), and we therefore defined effective neutralization as a greater-than-fivefold decrease in viral titer in a plasma sample. Both IgG1b12 and CD4-IgG2 neutralized HIV-1 in 5 of 6 plasma samples (Fig. 2). The degree of neutralization ranged from a 25- to a 625-fold reduction in the original infectious titer. In comparison, sCD4 and 19b, when used at the same concentration, neutralized HIV-1 in 0 of 4 and 2 of 6 plasma samples, respectively. Therefore, IgG1b12 and CD4-IgG2 appear to be more effective in neutralizing plasma HIV-1 isolates in ex vivo neutral-

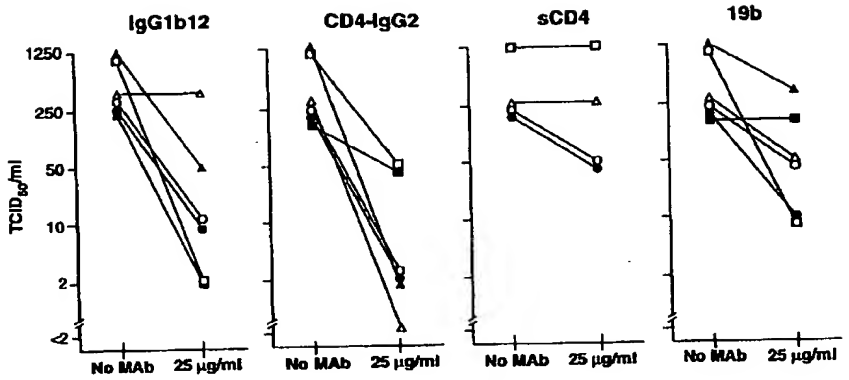

FIG. 2. Ex vivo neutralization of viruses from plasma samples by CD4-IgG2, IgG1b12, 19b, and sCD4. Each reagent (25 μg/ml) was incubated with serial dilutions of plasma samples from six HIV-1-infected patients. Viral replication was measured by the expression of p24 antigen in the culture supernatant on days 7 and 14, and an end-point titer was calculated. Each of the six HIV-1-infected patients is represented by a different symbol: △, patient no. 2; ▲, patient no. 3; ○, patient no. 8; ●, patient no. 9; □, patient no. 13; ■, patient no. 14. MAb, monoclonal antibody.

ization assays than are sCD4 and 19b. It is also important to note that viruses within one of the six plasma samples were resistant to neutralization by IgG1b12 but sensitive to CD4-IgG2 (patient no. 2), while viruses within another plasma sample (patient no. 14) were resistant to CD4-IgG2 but sensitive to IgG1b12. This result is not unexpected, considering that the two antibody preparations recognize distinct, though overlapping, sites on gp120 and that virus isolates that are resistant to one, the other, or both products have been previously described (41).

The ex vivo neutralization assay involves serially diluting patient plasma samples in 24-well tissue culture plates. Therefore, each well contains not only a different infectious inoculum of HIV-1 but also a different concentration of human plasma. To rule out the possibility that these different plasma concentrations could affect the ex vivo neutralization results, we tested the ability of IgG1b12 and CD4-IgG2 to neutralize virus present in four of the previous plasma samples when the plasma was diluted in culture medium or normal human plasma. Under the latter conditions, the concentration of human plasma was kept constant in all wells. As shown in Fig. 3, the use of plasma as a diluent did not affect the neutralization results by more than fivefold, indicating that high or low concentrations of plasma do not significantly affect the ex vivo neutralization assays. In addition, the results in these assays were similar to those shown in Fig. 1, indicating the reproducibility (within a fivefold difference) of the assays.

We next tested IgG1b12 and CD4-IgG2 at 1, 5, and 25 μg/ml in ex vivo assays. Results from seven different plasma samples are shown in Fig. 4. Viruses in some of the plasma samples (patients no. 301, 404, and 17) were sensitive to these antibody products at 1 μg/ml, while viruses in other plasma samples (patients no. 410 and 20) were sensitive only at 25 μg/ml. In general, however, it is remarkable how similar the activities of these two products against viruses in these seven plasma samples were. This would not be apparent, however, if only one concentration of antibody were tested (i.e., 25 μg/ml on plasma from patient no. 20). These results indicate that, in contrast to previous studies with sCD4 (10), effective ex vivo neutralization of virus in most plasma samples can be achieved with between 1 and 25 μg of IgG1b12 or CD4-IgG2 per ml.

To determine if passage of virus in plasma through PHA-activated PBMC would alter the neutralization sensitivity, in vitro neutralization assays against virus passaged once in PHA-activated PBMC (P1 isolates) recovered from six HIV-1-infected plasma samples were performed. Both reagents were utilized at graded concentrations from 0.001 to 100 μg/ml. In general, both IgG1b12 and CD4-IgG2 were active against most of the P1 isolates; the ID_{90} were <1 μg/ml for virus from patient no. 14, 1 to 10 μg/ml for patients no. 2 and 3, and 10 to 100 μg/ml for patients no. 17 and 20. Only virus from patient no. 9 could not be neutralized 90% or more by 100 μg of IgG1b12 or CD4-IgG2 per ml.

No correlation between the neutralization sensitivities of IgG1b12 and CD4-IgG2 in ex vivo assays and those in in vitro neutralization assays performed on P1 isolates from those plasma samples (Fig. 2, 4, and 5) was observed. As shown in Fig. 2, HIV-1 in the plasma from patient no. 2 was potently neutralized ex vivo by CD4-IgG2 (>625-fold at 25 μg/ml) and not by IgG1b12 (at the same concentration). However, the P1 isolate generated from that plasma was comparably neutralized in vitro by both reagents (ID_{50} and ID_{90}, 0.1 and 3.12 μg/ml, respectively [Fig. 5]). A further example is provided by a plasma sample from patient no. 14 that was not significantly neutralized in the ex vivo assay by CD4-IgG2 but was neutral-

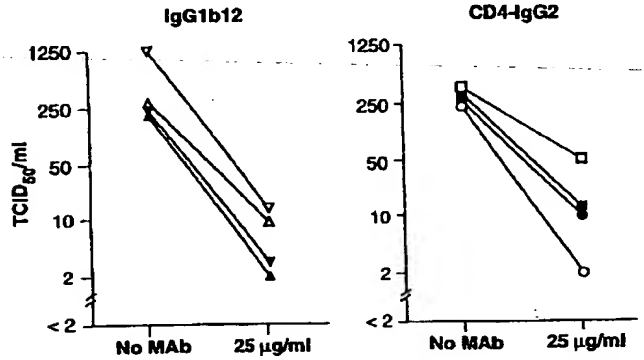

FIG. 3. Effect of normal human plasma on ex vivo neutralization. CD4-IgG2 and IgG1b12 were each incubated with two HIV-1-infected plasma samples diluted in medium (open symbols) or in normal human plasma (closed symbols). Viral replication was measured by the expression of p24 antigen present in the culture supernatants on days 7 and 14. Each of the four HIV-1-infected patients is represented by a different symbol: △, patient no. 3; ▽, patient no. 13; ○, patient no. 2; □, patient no. 14. MAb, monoclonal antibody.

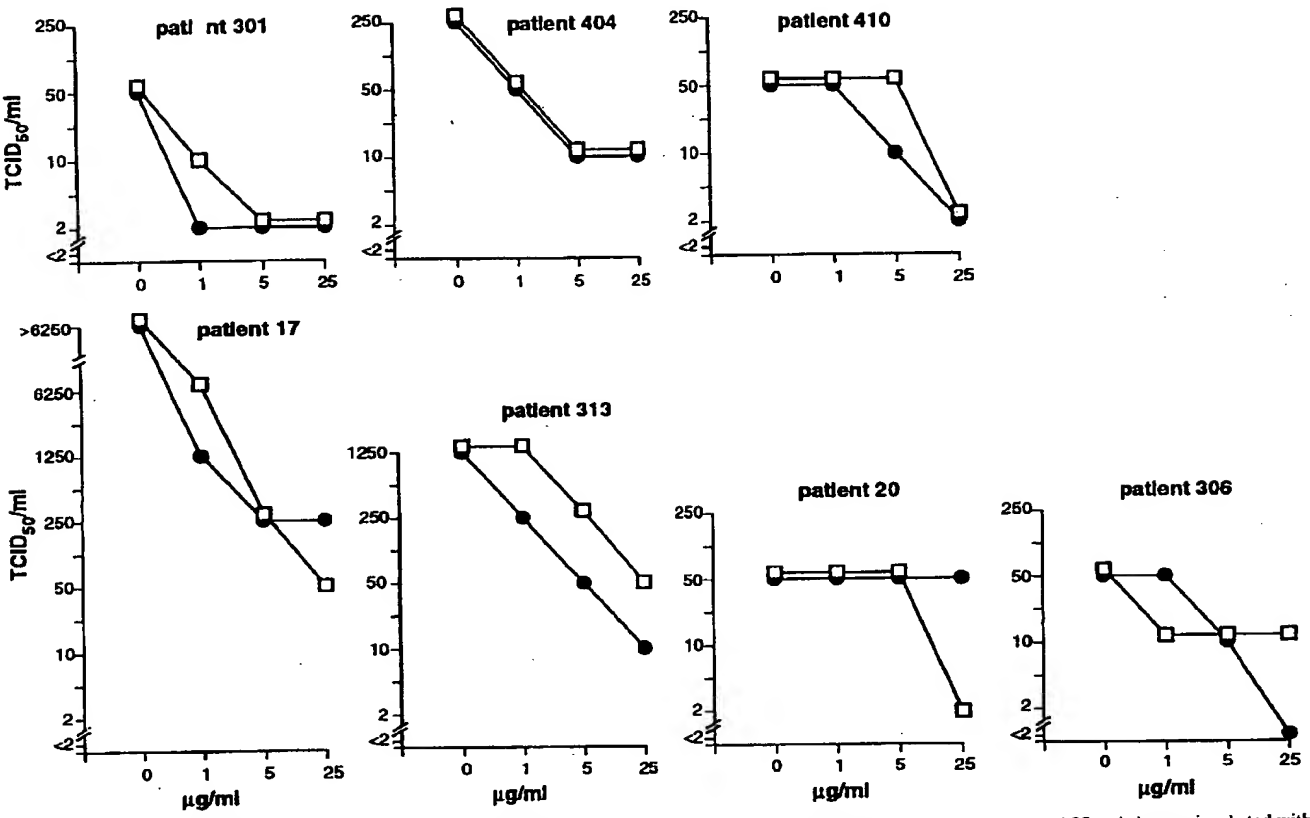

FIG. 4. Ex vivo neutralization at various concentrations of CD4-IgG2 and IgG1b12. CD4-IgG2 (□) and IgG1b12 (●), at 1, 5, and 25 µg/ml, were incubated with serial dilutions of plasma samples from seven HIV-1-infected patients. Viral replication was measured by the expression of p24 antigen in the culture supernatant on days 7 and 14, and an end-point titer was calculated.

ized by IgG1b12 (5- and 625-fold reduction in infectivity at 25 µg/ml, respectively [Fig. 2]), yet the corresponding P1 isolate was equally neutralized in vitro by both reagents (>95% neutralization at 25 µg/ml). Therefore, the viruses best neutralized in vitro were not necessarily derived from the plasma samples with the best ex vivo neutralization profiles. Much of this discrepancy may relate to selection of viruses upon passage in PHA-stimulated PBMC. The biphasic nature of many of the in vitro neutralization curves (IgG1b12 curve for patient no. 14, for instance [Fig. 5]) indicates that some of the P1 isolates contain mixtures of viruses with different sensitivities to IgG1b12 and CD4-IgG2.

Our results indicate that both human monoclonal antibody IgG1b12 and the CD4-based molecule CD4-IgG2 effectively neutralize HIV-1 directly from the plasma of seropositive donors in an ex vivo neutralization assay. Additionally, IgG1b12 and CD4-IgG2 effectively neutralize T-cell line-adapted strains and primary isolates of HIV-1. However, when viruses recovered from plasma samples were expanded in PBMC to produce virus stocks, the in vitro neutralization sensitivity of those plasma-derived stocks could not be predicted by the previous ex vivo neutralization assays.

Both IgG1b12 and CD4-IgG2 interact with the discontinuous CD4-BS on gp120 (1, 4, 33). In order to maintain the ability to bind CD4, certain as yet undefined structural features of this epitopic region remain conserved between virus isolates (29). It is therefore logical that the two broadly reactive neutralizing antibody-based products described here interact with this region. Since gp120 exists in multimeric form on the surface of the virus (6, 12, 34), the structural constraints upon the CD4-BS may also be apparent in the multimeric structure of the surface protein. The structural and functional differences in gp120s from different isolates of HIV-1 may not be measurable in assays based upon the monomeric form of this glycoprotein. Indeed, it has been shown that the CD4-BS of primary HIV-1 isolates from diverse geographic clades are conserved (26) and accessible to monoclonal antibodies when measured on gp120 monomers. The CD4-BS as expressed on multimeric gp120-gp41 complexes may therefore be significantly different from the domain as expressed on gp120 monomers (22, 34, 39). Assaying the neutralization sensitivity of unpassaged virus to monoclonal antibodies may be one method for measuring the functional differences in multimeric gp120.

Many other antibodies that interact with the CD4-BS on gp120 have failed to demonstrate the broad and potent neutralization of primary isolates of HIV-1 as demonstrated by IgG1b12 and CD4-IgG2 (24). This fact can probably be explained by the fact that most of those products bound well to monomeric gp120 but were not specifically capable of binding to multimeric gp120. It has now been shown that virus neutralization correlates broadly with monoclonal antibody binding to the multimeric form of gp120 (34, 39). IgG1b12 has been shown to bind well to multimeric gp120 (34). This observation

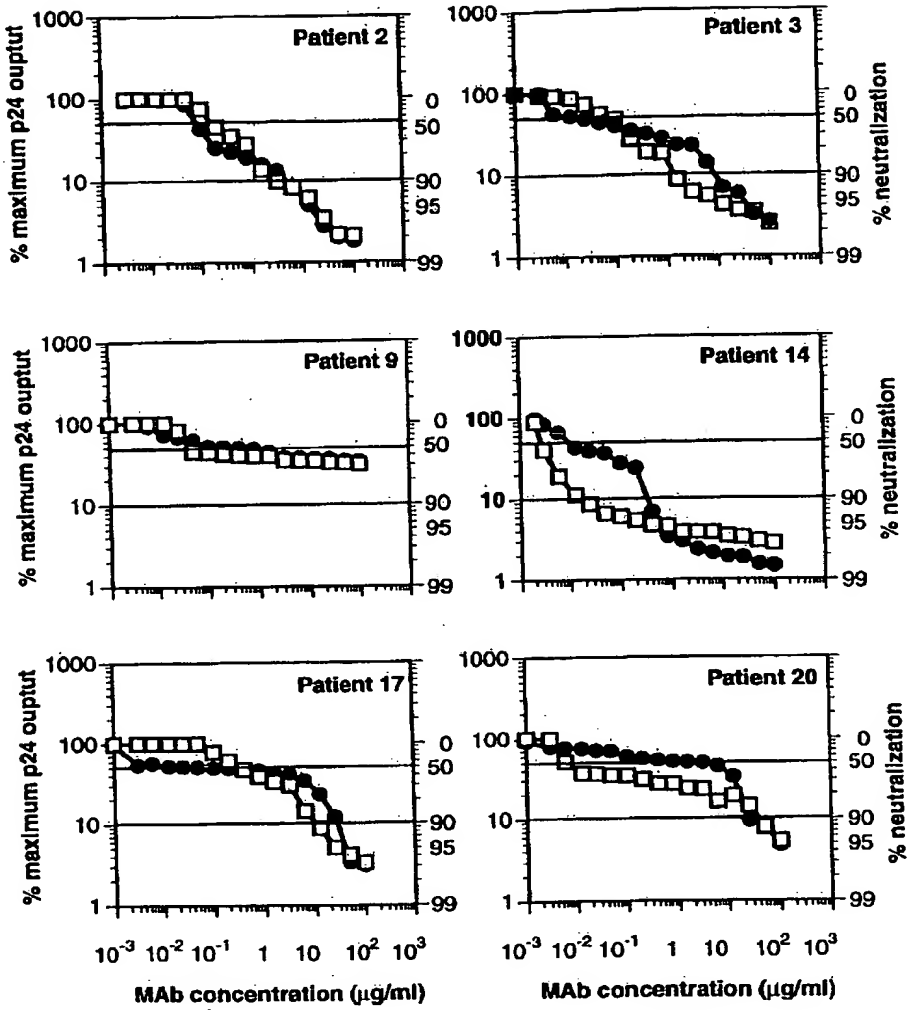

FIG. 5. In vitro neutralization of P1 isolates by CD4-IgG2 and IgG1b12. CD4-IgG2 (□) and IgG1b12 (●) were incubated at different concentrations with 200 $TCID_{50}$ of P1 isolates recovered from plasma samples. The virus replication was monitored by measurement of p24 antigen on days 5 to 8. Assays were run in triplicate, and the mean values were plotted as the percentage of maximum p24 output against input concentration (in micrograms per milliliter). Horizontal lines represent 50 and 90% inhibition of p24 output. MAb, monoclonal antibody.

probably explains its broad ability to neutralize primary HIV-1 isolates (7, 41).

Two observations make it unlikely that V3-specific antibodies (like 19b) will have the broad and potent neutralizing activity observed with IgG1b12 and CD4-IgG2. First, it is unlikely that a monoclonal product will overcome the amino acid sequence heterogeneity in this region (30). Second, while the V3 region may be well exposed on both oligomeric and monomeric forms of gp120 from T-cell line-adapted viruses, this region is shielded from monoclonal antibodies on the surface of primary isolates of HIV-1 (5).

We have demonstrated that there are differences in virus sensitivity to monoclonal products between ex vivo and in vitro neutralization assays. There are several potential explanations for this finding. During the process of expanding a P1 virus stock, selection of minor variants within the original sample may occur (21, 38). In this case, the virus species within the P1 stock will no longer accurately reflect the virus quasispecies present in the initial plasma sample. Since this expansion is performed in the absence of antibody-mediated immune pressure, it is not unreasonable to assume that a neutralization-sensitive virus, which would have been somewhat suppressed in vivo by the antibodies present in plasma, might rapidly grow to be the dominant species in the P1 isolate.

Factors other than selection of viral variants through in vitro passage may also be involved in the differences between the results of ex vivo and in vivo neutralization assays. The ex vivo assays are performed in the presence of plasma. The monoclonal products will therefore be affected by prebound antibodies which may compete for epitopes on the viruses. In addition, the plasma samples are not heated, so complement remains active. The ex viv assay may therefore also measure antibody-dependent complement-mediated neutralization that is not measured in the in vitro assay (37). Finally, since the P1 isolates are expanded in PHA-activated PBMC, they probably express higher levels of adhesion and class II molecules than

do viruses in plasma (2, 13, 15, 31). This may affect their sensitivity to neutralization.

The heterogeneity of gp120 among HIV-1 isolates and the lack of sensitivity of primary isolates of HIV-1 to neutralization have been major obstacles to vaccine development and the use of antibody-based therapeutic or prophylactic strategies (9, 10). The breadth and potency of CD4-IgG2 and IgG1b12 in neutralizing HIV-1 directly from plasma make them good candidates for future studies of HIV-1 prophylaxis in animal studies and in human trials.

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ARTICLES

Designing CD4 immunoadhesins for AIDS therapy

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A newly-constructed antibody-like molecule containing the gp120-binding domain of the receptor for human immunodeficiency virus blocks HIV-1 infection of T cells and monocytes. Its long plasma half-life, other antibody-like properties, and potential to block all HIV isolates, make it a good candidate for therapeutic use.

DESPITE the exquisite ability of the immune system to distinguish between self and non-self, and to put forth an impressive diversity in its antigen-recognizing repertoire, it can still be outflanked by a rapidly changing pathogen. Human immunodeficiency virus type 1 (HIV-1) is an example of such a pathogen, and, as a result, its consequences are devastating. Every individual infected with the virus is expected to develop a serious or life-threatening illness[1]; no protective state has been shown to be generated in natural infections. It has not yet been possible to generate a protective response by immunizing chimpanzees with gp120, the HIV-1 envelope glycoprotein[2,3], or to confer passive immunity to chimpanzees using human IgG[4]. Even neutralizing antibodies made in experimental animals can block the infectivity of only a few HIV-1 isolates[3,5]. Thus, the prospects for eliciting protective immunity against HIV-1, or for using antibodies as therapeutic agents to control HIV-1 disease are bleak. Anti-retroviral chemotherapy using dideoxynucleosides such as AZT does help some patients, but the toxicity is such that new strategies are needed[6].

We have therefore attempted to block HIV-1 infectivity with soluble derivatives of CD4, the receptor for HIV-1, with the rationale that the CD4-binding domain of gp120 is the only part of gp120 that the virus cannot afford to change[7]. CD4 is a cell-surface glycoprotein found mostly on a subset of mature peripheral T cells that recognize antigens presented by class II MHC molecules[8,9]. Antibodies to CD4 block HIV-1 infection of T cells[10,11] and human cells not susceptible to HIV-1 infection become so after transfection with a CD4 cDNA[12]. Gp120 binds CD4 with high affinity ($K_D \sim 10^{-9}$ M), suggesting that it is this interaction which is crucial to the entry of virus into cells[7,13]. Indeed, we[7] and others[14-18] have shown that soluble rCD4, lacking the transmembrane and cytoplasmic sequences of CD4, can block HIV-1 infectivity, syncytium formation, and cell killing by gp120 (ref. 19). rCD4 blocks the infectivity of diverse HIV-1 isolates (R.B., J.G., H.M. and S.B., unpublished results), and in theory should block all. At best, however, soluble rCD4 offers only a passive defence against the virus.

Active immunity requires a molecule such as an antibody, which can specifically recognize a foreign antigen or pathogen and mobilize a defence mechanism. Antibodies comprise two functionally independent parts, a rather variable domain (Fab), which binds antigen, and an essentially constant domain (Fc), providing the link to effector functions such as complement or phagocytic cells. It is almost certainly the lack of an antigen-binding domain which can neutralize all varieties of virus that hampers the development of humoural immunity to HIV-1. We reasoned that the characteristics of CD4 would make it ideal as the binding site of an antibody against HIV-1. Such an antibody would bind and block all HIV-1 isolates, and no mutation the virus could make, without losing its capacity to infect CD4+ cells specifically, would evade it. We therefore set out to construct such an antibody by fusing CD4 sequences to antibody domains.

We had two major aims for our hybrid molecules; first, as pharmacokinetic studies in several species predict that the half-life of soluble CD4 will be short in humans (30-120 min; J.M., unpublished results) we wished to construct a molecule with a longer half-life; second, we wanted to incorporate functions such as Fc receptor binding, protein A binding, complement fixation and placental transfer, all of which reside in the Fc portion of IgG. The Fc portion of immunoglobulin has a long plasma half-life, like the whole molecule, whereas that of Fab is short, and we therefore expected to be able to fuse our short-lived CD4 molecule to Fc and generate a longer-lived CD4 analogue. Because CD4 is itself part of the immunoglobulin gene superfamily, we expected that it would probably fold in a way that is compatible with the folding of Fc. We have therefore produced a number of CD4-immunoglobulin hybrid molecules, using both the light and the heavy chains of immunoglobulin, and investigated their properties. We have named one

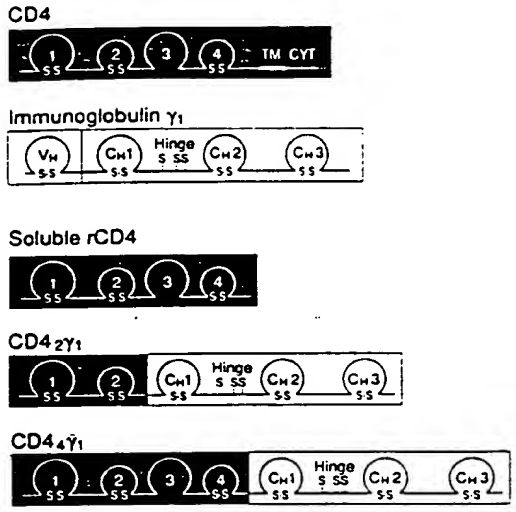

Fig. 1 Structure of cell surface CD4, human IgG1 (γ1), soluble rCD4, and CD4 immunoadhesins (2γ1 and 4γ1). The immunoglobulin-like domains of CD4 are numbered 1 to 4; TM and CYT refer to the transmembrane and cytoplasmic domains. Soluble rCD4 is truncated after proline 368 of the mature CD4 polypeptide. This results in a secreted, soluble polypeptide with an affinity for gp120 similar to that of cell surface CD4 (ref. 7). The vertical division within IgG1 indicates the junction of the variable (VH) and constant (CH1, hinge, CH2, and CH3) regions. Disulphide bonds formed within IgG1 domains and the immunoglobulin-like domains of CD4 are indicated by (S-S). The positions of cysteine residues that form intermolecular disulphide bridges connecting the IgG1 heavy-chain hinge to light and heavy chains are indicated by (S). CD4-derived and IgG1-derived domains of 2γ1 and 4γ1 are indicated by shaded and unshaded regions, respectively. The 2γ1 and 4γ1 immunoadhesins consist of residues 1 to 180 and residues 1 to 366 of the mature CD4 polypeptide, respectively, fused to the first residue (serine 114) of the human IgG1 heavy-chain constant region.

Methods. For the expression of CD4 immunoadhesins, the sequences of CD4 and human IgG1 were fused by oligonucleotide-directed deletional mutagenesis after their insertion into a mammalian expression vector used for soluble rCD4 expression[7]. A human IgG1 heavy-chain cDNA, obtained from a human spleen cDNA library using probes based on the published sequence[47], was inserted at a unique *Xba*I site found immediately 3′ of the CD4 coding region in the same reading orientation as CD4. Synthetic 48-mer oligodeoxynucleotides, complementary to the 24 nucleotides at the borders of the desired CD4 and IgG1 fusion sites, were used as primers in the mutagenesis reactions using the plasmid described above as the template[48].

particularly interesting class of these CD4-immunoglobulin hybrids 'immunoadhesins', because they contain part of an adhesive molecule[20] linked to the immunoglobulin Fc effector domain.

Synthesis of CD4 immunoadhesins

CD4 is an integral membrane protein with an extracellular region comprising four domains with homology to immunoglobulin variable domains[21,22] (Fig. 1). Soluble CD4 derivatives consisting of this extracellular region bind gp120 with the same affinity as cell-surface CD4 (ref. 7). CD4 variants containing only domains 1 and 2 also bind gp120[17,18], but the affinity of this interaction is not known. We constructed a series of hybrid molecules consisting of the first two or all four immunoglobulin-like domains of CD4 fused to the constant region of antibody heavy and light chains (Fig. 1).

We investigated the synthesis and secretion of these hybrids using transient expression in a human embryonic kidney-derived cell line. As shown in Fig. 2, immunoglobulin light and heavy chains are efficiently expressed in these cells, and light chain is efficiently secreted, but heavy chain is not unless a light chain is coexpressed. Thus the rules governing immunoglobulin chain secretion in these cells are the same as those for plasma or other lymphoid cells[23]. We first constructed hybrids that fused CD4 with the constant regions of murine κ- or γ1-chains. These hybrids contained either the first two or all four immunoglobulin-like domains of CD4, linked at a position chosen to mimic the spacing between disulphide-linked cysteines seen in immunoglobulins (Fig. 1). As expected, the CD4-κ hybrids were secreted well, whereas hybrids between CD4 and mouse γ1-chain were expressed but not secreted unless a κ-chain or a CD4-κ hybrid was present.

A different and unexpected picture emerged when analogous CD4-heavy-chain hybrids were constructed using the constant region of human IgG1 heavy chain instead of mouse heavy chain. Such hybrids, containing either the first two or all four immunoglobulin-like domains of CD4 (named 2γ1 and 4γ1 respectively), were secreted in the absence of wild-type or hybrid light chains (Fig. 2*a*). Both 2γ1 and 4γ1 could be directly immunoprecipitated using *Staphylococcus aureus* protein A, which binds the Fc portion of IgG1, indicating that the protein A-binding sites of these constructs are fully functional. Indeed, both molecules can be purified to near homogeneity on protein A columns (Fig. 2*b*).

Structure of CD4 immunoadhesins

We examined the subunit structure of these immunoadhesin molecules using SDS-polyacrylamide gels (Fig. 2*b*). Without any reducing agent, the apparent relative molecular mass (M_r) of each construct doubled, demonstrating that both immunoadhesins are disulphide-linked dimers. The hinge region of each immunoadhesin contains three cysteine residues, one normally involved in disulphide bonding to light chain, the other two in the intermolecular disulphide bonds between the two heavy chains in IgG. As the molecules are dimers at least one, and perhaps, all three, of these cysteine residues are involved in intermolecular disulphide bonds. We examined the capacity of 2γ1 and 4γ1 to form disulphide links with light chains. When an immunoadhesin construct was cotransfected with a light chain, the light chain produced could be precipitated by protein A. Mutagenic substitution of the first hinge-region cysteine with alanine abolished light-chain bonding, but did not affect dimerization (data not shown), indicating that this cysteine bonds the light chain in these hybrids, as in normal IgG. Thus the disulphide bond structure of these immunoadhesins seems to be analogous to that of immunoglobulins.

gp120 binding

To determine whether our immunoadhesins retain the ability to bind gp120 with high affinity, and whether the first two immunoglobulin-like domains are sufficient, we carried out saturation binding analyses with radioiodinated gp120. Binding is saturable, showing a simple mass action curve (Fig. 3*a*). The dissociation constant (K_d) for the interaction of each immunoadhesin with gp120, calculated by Scatchard analysis (Fig. 3*a*, inset), was indistinguishable from that of soluble rCD4 ($\sim 10^{-9}$ M) (Table 1). Thus, the N-terminal 170 amino acids of CD4 are sufficient for high-affinity binding. As these immunoadhesins are homodimeric, they should each have two gp120 binding sites. We examined this possibility by coating plastic microtitre wells with gp120, then adding soluble CD4 or immunoadhesins. Both immunoadhesins could bind added labelled gp120, whereas soluble rCD4, with only one gp120 binding site, could not (J. Porter and S. C., unpublished results). To confirm the bivalent nature of 2γ1 and 4γ1, we examined their ability to agglutinate sheep red blood cells coated with gp120. Again, both CD4 immunoadhesins, but not soluble rCD4, agglutinated the cells, showing that binding to gp120 molecules on different cells is not sterically hindered.

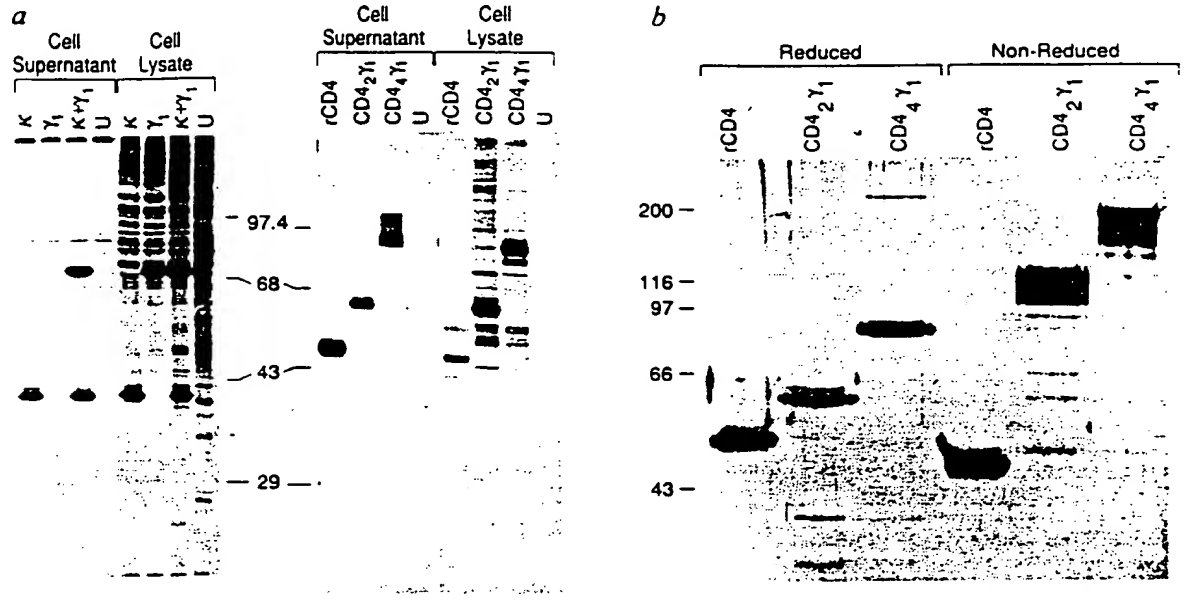

Fig. 2 Expression, secretion and subunit structure of CD4 immunoadhesins and soluble rCD4. *a*, Expression and secretion of mouse immunoglobulins, soluble rCD4 and CD4 immunoadhesins expressed in mammalian cells. Cells were transfected with vectors directing the expression of murine κ-light chain (lanes κ) or γ1-heavy chain (lanes γ1) individually or together (lanes κ + γ1), vectors encoding soluble rCD4 (lanes rCD4), and the CD4 immunoadhesins 2γ1 (lanes $CD4_2\gamma_1$) or 4γ1 (lanes $CD4_4\gamma_1$). After metabolic labelling with [^{35}S]methionine, cell supernatants and cell lysates were analysed by immunoprecipitation. Lanes U, untransfected cells. *b*, Subunit structure of secreted CD4 immunoadhesins and soluble rCD4. Soluble rCD4, 2γ1 and 4γ1 were purified from culture supernatants of transfected cells and analysed by electrophoresis on a 7.5% SDS-polyacrylamide gel. Samples were prepared in buffer with 10 mM dithiothreitol (DTT) (reducing conditions) or without DTT (non-reducing conditions). The positions of relative molecular mass standards are indicated (in thousands). Both immunoadhesins behaved as disulphide-linked dimers; in contrast, soluble rCD4 which is monomeric, displayed only a minor change in mobility upon reduction of its intra-molecular disulphide bonds.

Methods. *a*, Cells were transfected by a modification of the calcium phosphate procedure, labelled with [^{35}S]methionine, and cell lysates prepared as described[19]. Immunoprecipitation analysis was carried out as previously described[7], with the exception that no preadsorbtion with Pansorbin (Calbiochem) was done, and the precipitating antibodies used were 2 μl of rabbit anti-mouse IgG serum (Cappell) for mouse IgG heavy and light chains, 0.25 μg of OKT4A (Ortho) for soluble rCD4, and no added antibody (Pansorbin only) for the CD4 immunoadhesins. Immunoprecipitated proteins were resolved on 10% SDS-PAGE gels, and visualized by autoradiography. *b*, CD4 immunoadhesins were purified from transfected cell supernatants by protein A affinity chromatography followed by ammonium sulphate precipitation. Purified proteins were subjected to SDS-PAGE under both reducing and non-reducing conditions and visualized by silver staining.

In vivo plasma half-life

We examined whether the immunoadhesins share the long *in vivo* half-life of antibodies. Studies of rCD4 in rabbits provide clearance data that extrapolate well to other species, including humans (J.M., unpublished results). The change in plasma concentration with time for each of the three CD4 analogues in rabbits is shown in Fig. 4. Analysis of these data reveals that soluble rCD4 has a terminal half-life in rabbits of ~15 min, whereas 4γ1 and 2γ1 have terminal half-lives of ~7 and 48 h, respectively (Table 1). Thus the half-life of 2γ1 in rabbits is nearly 200 times longer than that of rCD4 and comparable to that of human IgG in rabbits (4.7 days)[24]. The half-life of 2γ1 in humans is expected to be longer than that in rabbits, because of the decreased proportional blood flow to eliminating organs as species increase in size[25], and should be comparable with that of human IgG1 (21 days).

Our results confirm our initial hypothesis that, as in the case of immunoglobulin itself, one can increase the stability of a rapidly cleared molecule (Fab or rCD4) by fusing it to a long-lived molecule, Fc. The swift clearance of rCD4 is probably largely due to its size, M_r 55,000, which means it is just small enough to be cleared efficiently by renal filtration. One component in the increased half-lives of these molecules is therefore probably their larger size; but this cannot be the whole story as 4γ1, although larger than 2γ1, has a shorter half-life. Both 4γ1 and rCD4, but not 2γ1, contain two CD4-derived Asn-linked carbohydrate sites which are glycosylated in rCD4 (R. Harris and M. Spellman, unpublished results); these sugar moieties

Table 1 Properties of CD4 immunoadhesins and soluble rCD4

	Calculated M_r	Subunit structure	gp120 binding (nM)*	Blocks infectivity T cells	Blocks infectivity Mθ	Plasma half-life in rabbits (hours)†	Fc binding (nM)*	Complement binding	Protein A binding
rCD4	41,000	monomer	2.3 ± 0.4	Yes	Yes	0.25 ± 0.01	—	No	No
4γ1	154,000	dimer	1.2 ± 0.1	Yes	Yes	6.7 ± 1.1	2.3 ± 0.7	No	Yes
2γ1	112,000	dimer	1.4 ± 0.1	Yes	Yes	48.0 ± 8.6	2.6 ± 0.3	No	Yes
IgG1	146,000	tetramer (H_2L_2)	—	—	—	113‡	3.2 ± 0.2	Yes	Yes

* Standard error of the mean was determined using the Inplot and Scatplot programs (see Fig. 3 legend). † Standard deviation indicated in hours.
‡ Determined in ref. 24 (IgG1 has a half-life of 21 days in humans).

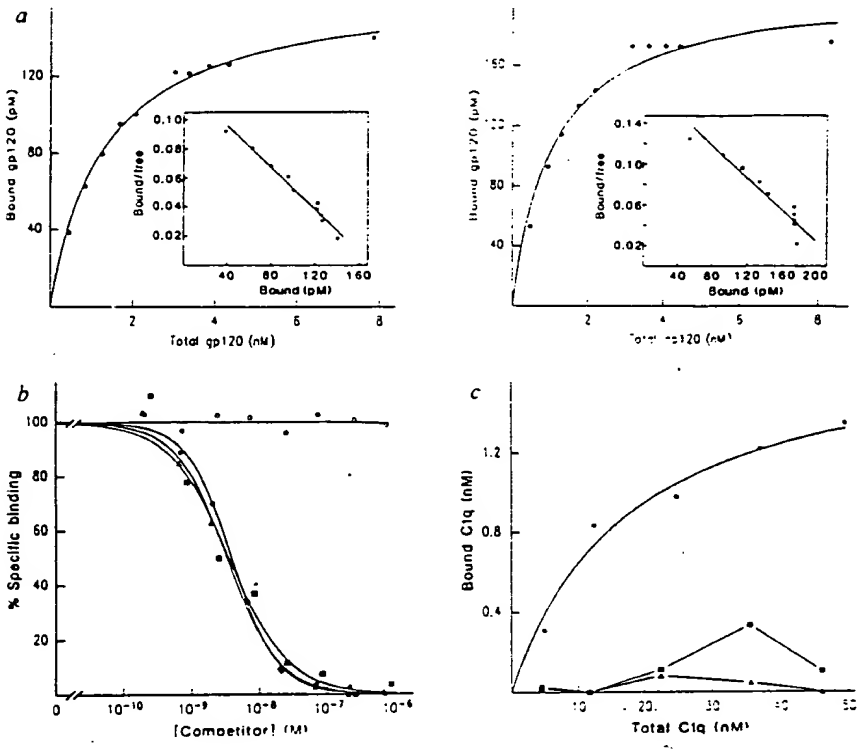

Fig. 3 Binding properties of CD4 immunoadhesins. *a*, Gp120 saturation binding analysis of CD4 immunoadhesins. Immunoadhesin proteins 4γ1 (left) or 2γ1 (right) in transfected cell supernatants were incubated with increasing concentrations of purified soluble rgp120 (ref. 50) radioiodinated with lactoperoxidase. The lines drawn for the binding curves and for the Scatchard plots of the data (shown in the insets) represent the best fit as determined by unweighted least-squares linear regression analysis. Dissociation constants calculated from these results and from binding studies of gp120 to soluble rCD4 performed in parallel are given in Table 1. *b*, Binding of CD4 immunoadhesins to Fcγ receptors on U937 cells. Competition binding analysis was carried out by mixing 0.1 μg ml^{-1} of ^{125}I-labelled human IgG1 (Calbiochem) with increasing concentrations of purified human IgG1 (solid circle), 2γ1 (solid square), 4γ1 (solid triangle), or soluble rCD4 (open circle) proteins. Curves drawn represent the best fit as determined by unweighted least-squares nonlinear (IgG1, 2γ1 and 4γ1) or linear (rCD4) regression analysis. Dissociation constants calculated from these results are shown in Table 1. *c*, C1q saturation binding analysis of CD4 immunoadhesins. Purified anti-gp120 IgG2a mouse monoclonal antibody (solid circle), 2γ1 (solid square), or 4γ1 (solid triangle) proteins were aggregated by binding to gp120-coupled Sepharose, and incubated with increasing concentrations of purified human C1q (Calbiochem) radioiodinated with lactoperoxidase. The curve drawn for the anti-gp120 monoclonal antibody (mAb) represents the best fit as determined by least-squares nonlinear regression analysis; the dissociation constant for C1q binding to this gp120-aggregated anti-gp120 mAb was $\sim 1.8 \times 10^{-8}$ M.

Methods. *a*, Gp120 saturation binding analysis was carried out as described[7] except that gp120-CD4 immunoadhesin complexes were collected directly onto Pansorbin: binding was comparable to that observed when complexes were collected with OKT4A as for soluble rCD4. Specifically bound ^{125}I-labelled gp120 was determined from the difference in binding in the presence or absence of a 1,000-fold excess of unlabelled rgp120 and is plotted against the total ^{125}I-labelled gp120 concentration. *b*, FcR binding analysis was done essentially as described[27] except that after centrifugation free IgG1 was removed by aspiration of the aqueous and oil layers. Mixtures of ^{125}I-labelled human IgG1 and IgG1, CD4 immunoadhesins or soluble rCD4 were incubated with U937 cells (2×10^6 cells per tube) for 60 min at 4 °C. Specific binding was calculated by subtracting residual nonspecific binding (<25% of specific binding) which could not be competed out by a 1,000-fold excess of unlabelled human IgG1. *c*, C1q binding analysis was done essentially as described[29], except that gp120 coupled to CNBr-activated Sepharose 6B (Pharmacia) was used as the solid support to aggregate CD4 immunoadhesins or the anti-gp120 mouse mAb. Proteins were adsorbed to gp120 coupled-beads, incubated with varying concentrations of ^{125}I-labelled C1q, and bound and free C1q were then separated by centrifugation through 20% sucrose. Specific binding was determined from the difference in binding in the presence or absence of added antibody or immunoadhesin. All data analysis was carried out using the Inplot and Scatplot programs (R. Vandlen, Genentech). Scatplot was modified from the Ligand program (P. Munch, NIH).

may facilitate clearance by receptors in the liver. The charge of the molecule may also be important, as the CD4 portion of 4γ1 contributes a net excess of eleven positively charged amino acids on 4γ1, but only three on 2γ1. This may increase uptake of rCD4 and 4γ1 onto anionic surfaces, accelerating their clearance from the circulation.

Fc receptor and complement binding

Two major mechanisms for the elimination of pathogens are mediated by the Fc portion of specific antibodies. Fc activates the classical pathway of complement, ultimately resulting in lysis of the pathogen, whereas binding to cell Fc receptors can lead to ingestion of the pathogen by phagocytes or lysis by killer cells. The binding sites for Fc cell receptors and for the initiating factor of the classical complement pathway, C1q, are found in the constant region of heavy chain[26] (the CH2 domain for C1q[27] and the region linking the hinge to CH2 for Fc cell receptors[28]). We aimed to incorporate both of these functions into the immunoadhesins. We chose the IgG1 subtype to supply the Fc domain because IgG1 is the best compromise between Fc binding, C1q binding, and long half-life. We show below that the immunoadhesins bind FcR well, but do not bind C1q.

Three types of Fc cell receptors are known to be expressed on a variety of leukocytes. Of these FcRI, principally expressed on mononuclear phagocytes, is the only one which binds monomeric human IgG1 with high affinity[26]. We used competition binding analysis with FcRI receptors on the U937 monocyte/macrophage cell line to characterize the Fc receptor binding of 2γ1 and 4γ1. Direct saturation binding analysis with human IgG1 gave a K_d of $\sim 3 \times 10^{-9}$ M. In competition binding analyses, the two CD4 immunoadhesins, but not rCD4, bound to Fc receptors on U937 cells to the same extent and with an affinity indistinguishable from human IgG1 (Fig. 3*b*, Table 1).

We examined the ability of the immunoadhesins to bind to the first component of the classical pathway of complement, C1q, by saturation binding analysis. Because binding of C1q increases with the aggregation state of the antibody, with an affinity of $\sim 10^{-4}$ for monomers and $\sim 10^{-8}$ for tetramers of IgG[26], we first aggregated the immunoadhesin using gp120 linked to Sepharose. As a positive control, we measured C1q binding to an anti-gp120 mouse IgG2a monoclonal antibody, (which like human IgG1 binds C1q with high affinity[29]) aggregated by the same gp120-Sepharose. The affinity of the mouse antibody for C1q determined by Scatchard analysis was 1.8×10^{-8} M (Fig. 3*c*), comparable to that observed for other mouse IgG2a and for human IgG1 antibodies. In contrast, neither immunoadhesin bound C1q to any detectable extent (Fig. 3*c*),

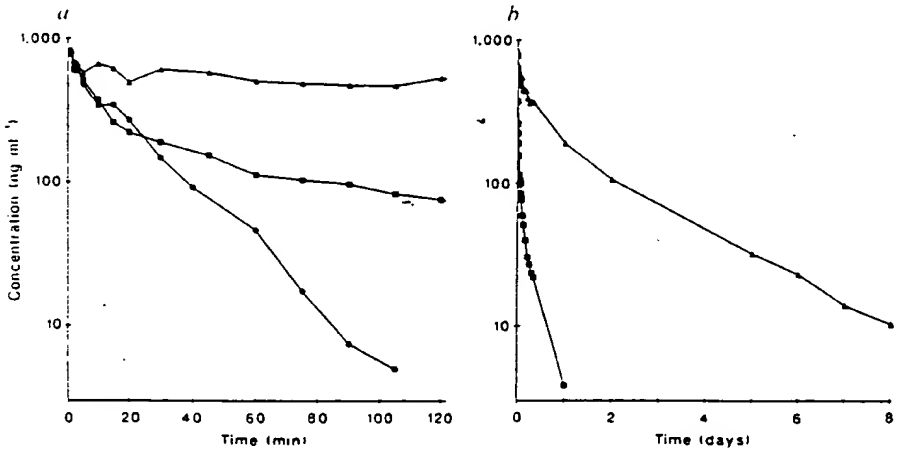

Fig. 4 Pharmacokinetics of CD4 immunoadhesins and soluble rCD4. Shown are the mean plasma concentrations (ng ml^{-1}) for 2γ1 (triangles), 4γ1 (squares), and rCD4 (circles) following a single intravenous administration in rabbits. *a*, Time course of plasma clearance over the first 120 minutes; *b*, time course over 8 days after injection of the CD4 analogues. **Methods.** Ten female New Zealand white rabbits (Rabbitek, Modesto, California) were injected intravenously (via an ear vein catheter) with a single bolus dose (40 μg kg^{-1} in a volume of 1 ml) of either rCD4 ($n = 2$), 4γ1 ($n = 4$), or 2γ1 ($n = 3$). Blood samples were obtained from an arterial catheter in the opposite ear; after 24 hours, blood samples were obtained by venipuncture. Plasma concentrations of each protein were determined by an enzyme-linked immunosorbent assay. This capture assay used two antibodies, including an anti-CD4 monoclonal directed against the gp120-binding site (and capable of blocking gp120 binding), and thus provided a sensitive assay for CD4-containing molecules that are still capable of binding gp120. Exponential equations were fitted to the data of individual rabbits using a nonlinear least squares regression program NONLIN84[35] (Statistical Consultants, Lexington, Kentucky). The concentration (C, ng ml^{-1}) versus time (t) data for rCD4 were best described by a biexponential equation $C = 541\,e^{-1.34t} + 620\,e^{-0.0472t}$ where time is in minutes; the average terminal half-life was 14.7 min, and the average clearance was 3 ml min^{-1} kg^{-1}. The 4γ1 data were best described by a triexponential equation, $C = 546\,e^{-211t} + 193\,e^{-20.5t} + 46.8\,e^{-2.54t}$, where time is in hours. The average terminal half-life was 6.7 hours, and the average clearance was 0.91 ml min^{-1} kg^{-1}. The 2γ1 data were best described by a triexponential equation, $C = 153\,e^{-53.2t} + 342\,e^{-2.19t} + 183\,e^{-0.351t}$, where time is in hours. The average terminal half-life was 48 hours, and the average clearance was 0.039 ml min^{-1} kg^{-1}.

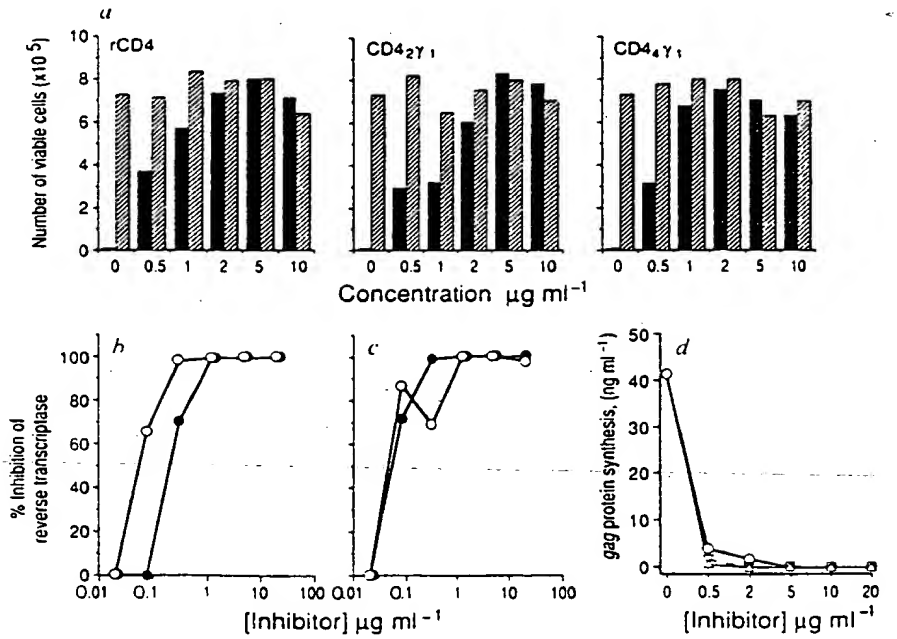

Fig. 5 Inhibition of HIV-1 infectivity by CD4 immunoadhesins and soluble rCD4. *a*, Inhibition of the cytopathic effects on ATH8 cells by HIV-1 was examined as described[32] with the HTLV-IIIB isolate[31]. The number of viable cells at day 10 after infection is shown for varying concentrations of each molecule in the presence (solid bars) or absence (shaded bars) of added virus. The absence of an effect of each CD4 analogue on cell number in the absence of virus indicates that none of these molecules inhibited cell growth. *b*, Inhibition of infection of H9 cells by HIV-1 was carried out as described[7] with the HTLV-IIIB isolate. Reverse transcriptase activity was determined 7 days after infection and is given as the percentage of the level seen in the absence of inhibitor. Solid and open circles represent 2γ1 and 4γ1, respectively. *c*, Inhibition of infection of U937 cells by HIV-1 (HTLV-IIIB isolate) was carried out as described above for H9 cells. *d*, Inhibition of infection of fresh human monocytes by the monocytotropic HIV-1 isolate Ba-L (ref. 35). HIV-1 replication was determined by measuring the level of p24 gag antigen synthesis 10 days after infection using a commercial assay kit (Dupont). Circles, inverted triangles and triangles represent inhibition of p24 synthesis by soluble rCD4, 2γ1 and 4γ1, respectively.

lthough both did bind the gp120-Sepharose matrix in amounts omparable to the control antibody.

Thus, our immunoadhesins bind well to Fc receptors. It is erhaps surprising that they do not bind C1q. As far as is known, ll the critical contact residues for C1q binding reside in the H2 domain of the heavy chain[26], and are conserved among ll the human IgG isotypes. However, these have varying bilities to mediate complement fixation. Thus steric hindrance r other aspects of protein conformation (for example, the gmental flexibility of antibodies[30]) may be important.

nfectivity studies

wo systems were used to study the *in vitro* ability of CD4 mmunoadhesins to block infection of CD4-bearing T cells by the HIV-1 T-lymphotrophic isolate HTLV-IIIB (ref. 31). Infection with HIV-1 exerts a profound cytopathic effect on the human T-cell clone ATH8, with more than 98% of the cells being killed by day 10 after infection[32] (Fig. 5*a*). Both CD4 immunoadhesins blocked cell killing with the same potency as soluble rCD4, without inhibiting cell proliferation; each CD4 analogue completely abolished cell killing at a concentration of ~0.05 μM (Fig. 5*a*). Complete protection was also observed at comparable concentrations with a different HIV-1 isolate, HTLV-III RF, which is not neutralized by sera from animals immunized with rgp120 from the IIIB isolate[5]. We also examined the production of HIV-1 reverse transcriptase activity after infection of the H9 human T-cell line. Again, both immunoadhesins completely blocked virus production by day 7 (Fig. 5*b*), at

concentrations comparable to rCD4 (data not shown); moreover the potency of each CD4 analogue was markedly higher (~fivefold) than that observed in the ATH8 assay.

Monocyte infection

Because it has been suggested that antibodies present in sera from HIV-1 infected individuals may enhance the infectivity of HIV-1 in Fc receptor (FcR)-bearing cells such as primary blood monocytes[33], and monocyte cell lines[34], we examined the effect of rCD4 and CD4 immunoadhesins on HIV-1 infection of FcR-expressing cells of monocyte/macrophage origin. Both CD4 immunoadhesins completely blocked HIV-1 IIIB virus production in U937 cells at similar concentrations to those found to be effective on H9 cells (Fig. 5*c*), with a potency comparable to that of soluble rCD4 (data not shown). In another system, the replication of a monocytotrophic HIV-1 isolate, Ba-L[35], in fresh monocytes was monitored by the production of p24 antigen. Soluble rCD4 completely blocked infection, indicating that infection of monocytes by the Ba-L isolate does involve CD4. Both CD4 immunoadhesins also completely blocked p24 production, at concentrations equal to or lower than rCD4 (Fig. 5*d*). Thus the CD4 immunoadhesins are at least comparable to soluble rCD4 in their ability to prevent infection of monocyte/macrophages by HIV; no evidence was found for enhancement of infection by immunoadhesins (or by soluble rCD4) in cells which express high affinity Fc receptors.

Implications for treatment of HIV-1 disease

Because the hallmark of HIV-1 disease is the specific destruction of $CD4^+$ T cells, and the progression of infected individuals to AIDS closely parallels their decline in $CD4^+$ T-cell number[36], it is reasonable to believe that the interaction of gp120 with CD4, either by direct HIV-1 infection of $CD4^+$ cells or otherwise, underlies the killing of $CD4^+$ cells. Therefore, if this interaction can be stopped it may be possible to prevent disease progression. But despite the logic of this hypothesis, the observation that only a very few lymphocytes are actively infected with HIV-1 *in vivo*[37] has posed a problem to those attempting to explain the causative role of HIV-1 in the aetiology of AIDS[38]. Two observations may explain the 'catalytic' ability of HIV-1 to deplete $CD4^+$ lymphocytes: first, a single infected cell can fuse many uninfected $CD4^+$ cells to itself, creating an inviable mass[39,40]; and second, gp120 is shed from the surface of HIV-1-infected cells and virions[41], as its link to gp41, its anchor protein partner, is probably non-covalent. This shed gp120 binds to surface CD4 on uninfected cells with high affinity, and can result in their functional alteration[42,43] or death by one of two pathways shown to operate *in vitro*. Bystander cells coated with gp120 bound to their CD4 surface molecules become targets for anti-gp120 antibodies produced by HIV-1 infected individuals and can be killed via antibody-dependent cell-mediated cytotoxicity[44]. Also, MHC class II-positive $CD4^+$ T cells can internalize gp120 bound tightly to CD4 on their surface, process it, and present peptides derived from it on their class II molecules, thus becoming sensitive, even at low gp120 concentrations, to lysis by gp120-specific cytotoxic T cells[19,45,46]. The important common factor in all these proposed mechanisms of cell destruction is that gp120 must bind specifically to cell-surface CD4. If these mechanisms are important *in vivo*, this would imply that soluble rCD4 could intervene.

But to affect the disease noticeably, one would expect to need to maintain a high concentration of rCD4, which is hampered by its rapid clearance. Our approach to this problem was to fuse the gp120-binding domain of CD4 to a molecule well designed to avoid the clearance mechanisms of the body. Indeed, the Fc domain and CD4 sequences are structurally compatible, as the hybrid molecules have important properties of both parents. Thus, they bind gp120 and block infection of T cells by T-lymphotrophic HIV-1 and of monocytes by monocytotrophic HIV-1. They are also comparable to antibodies in their long plasma half-life and their ability to bind Fc receptor and protein A. This combination of properties allows both better passive defence, due to the higher plasma concentration attainable even with infrequent injection, and the possibility o actively attacking HIV-1 and infected cells. A high steady-stat level also makes it more likely that effective concentrations wi be attained in lymph and lymphatic organs, where HIV may b most active.

The high-affinity binding of the immunoadhesins to Fc recep tors implies that mechanisms of pathogen elimination, such a phagocytic engulfment and killing by antibody-dependent cell mediated cytotoxicity, may be recruited by these immuno adhesins to kill HIV-1 infected cells and virus. As it is possibl that antibody-dependent cell-mediated cytotoxicity in an infec ted individual may be more a mechanism of pathology in HIV- infection than a protective response[44], it is important to note difference between CD4 immunoadhesins and the patients' owr anti-gp120 antibodies: the immunoadhesin, in contrast to anti body, cannot recognize gp120 bound to an uninfected $CD4^-$ bystander cell, as gp120 has only a single binding site for CD4 Because placental transfer of antibody, unique to the IgG sub class, also proceeds through an FcR-dependent mechanism CD4 immunoadhesins may also be transferred *in utero*. This may have implications for the prevention of perinatally transmit ted HIV-1 infection.

Although it is not yet clear which of the functions o immunoglobulins will be advantageous when applied to HIV infection, we have taken the approach of trying to add al possible functions to our immunoadhesins. Once the structura requirements for the optimal molecule are established, function can be tailored at will, as the parent antibody molecule is sc well understood.

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LETTERS TO NATURE

A 110-ms pulsar, with negative period derivative, in the globular cluster M15

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We report the discovery of a 110-ms pulsar, PSR2127+11, in the globular cluster M15 (NGC7078)[1]. The results of nine months of timing measurements place the new pulsar about 2″ from the centre of the cluster, and indicate that it is not a member of a close binary system. The measured negative value of the period derivative, $\dot{P} \approx -2 \times 10^{-17}$ s s^{-1}, is probably the result of the pulsar being bodily accelerated in our direction by the gravitational field of the collapsed core of M15. This apparently overwhelms a positive contribution to $\dot{P}$ due to magnetic braking. Although PSR2127+11 has an unexpectedly long period, we argue that it belongs to the class of 'recycled' pulsars, which have been spun up by accretion in a binary system. The subsequent loss of the pulsar's companion is probably due to disruption of the system by close encounters with other stars[2,3].

The discoveries of millisecond pulsars in globular clusters M28 (ref. 4) and M4 (ref. 5) led us to survey all clusters accessible to the 305-m Arecibo radio telescope ($0° \leq \delta \leq 38°$). A dual-polarization, 40-MHz-bandwidth signal at 1415 MHz was passed through the Arecibo digital correlator, sampled with 128 lags every 506.6 μs, and recorded on tape. The relatively high central radio frequency ensured an almost interference free signal and minimized the effects of interstellar dispersion and scattering, which can be significant for distant, low-galactic-latitude clusters. M15 was observed on 28 December 1987 for 90 minutes, which corresponds to ~11 million samples.

The data were analysed at both the Cornell National Supercomputer Facility (IBM 3090-600E) and the Los Alamos National Laboratory (Cray X-MP). Both analyses involved preliminary dedispersion of the multichannel data at 128 or 64 trial dispersion measures, followed first by one-dimensional Fourier transformation of the dedispersed time series and then by a search for harmonically related spikes in the resultant power spectra. The Cray X-MP analysis used the full, 11-million-sample data arrays to obtain maximum sensitivity with regard to isolated pulsars. The data analysed with the IBM supercomputer were divided into five 2-million-sample blocks, which were treated separately to maintain high sensitivity to binary pulsars with short orbital periods. The nominal 6σ sensitivities of these two analysis schemes were 0.05 mJy and 0.1 mJy respectively, for the periods down to ~2.5 ms.

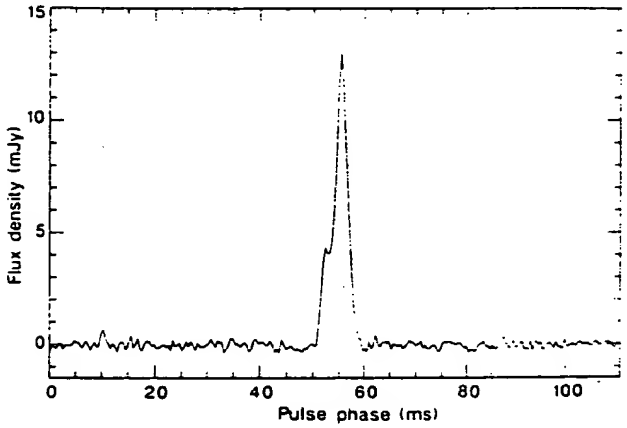

Fig. 1 The average pulse profile of PSR2127+11 at 1415 MHz. The effective resolution is ~800 μs and the integration time is 7 hours.

The data analysis at Cornell revealed the presence of a 110-ms, high-Q periodicity in the received signal with dispersion measure DM ≈ 60 pc cm^{-3}. This detection was subsequently confirmed at Los Alamos. Further observations made at Arecibo on 20 and 21 February 1988 confirmed the discovery of a 110-ms pulsar. The average pulse profile of PSR2127+11 observed at 1415 MHz is shown in Fig. 1. The pulsar parameters, derived from our twice-weekly timing observations over nine months, are summarized in Table 1. Errors quoted are the standard 3σ errors of a model fit to the observed pulse arrival times.

Although the precise timing and Very Large Array (VLA) positions of PSR2127+11 will become known soon, the present positional accuracy is sufficient to conclude that the pulsar is located well within the 6″ core radius of the cluster, 2.0″ west and 0.6″ north of the centre[6]. The dispersion measure of PSR2127+11, DM = 67.25 pc cm^{-3}, agrees well with that expected from a simple model of the galactic electron density distribution[7], given the distance, D = 9.7 kpc, and galactic coordinates,

Table 1 Measured parameters of the pulsar PSR2127+11

Pulsar period	0.11066470954 ± 0.00000000001 s
Period derivative	$(-20 \pm 1) \times 10^{-18}$ s s^{-1}
Epoch	JD 2447213.15
Dispersion measure	67.25 ± 0.05 pc cm^{-3}
Flux density (430 MHz)	1.7 ± 0.4 mJy
Flux density (1400 MHz)	0.2 ± 0.05 mJy
Right Ascension (B1950.0)	21^{h}27^{m}33.22^s ± 0.01
Declination (B1950.0)	11°56′49.4″ ± 0.3
Distance	9.7 kpc

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